# Two roles for the *Drosophila* IKK complex in the activation of Relish and the induction of antimicrobial peptide genes

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**The *Drosophila* NF-κB transcription factor Relish is an essential regulator of antimicrobial peptide gene induction after Gram-negative bacterial infection. Relish is a bipartite NF-κB precursor protein, with an N-terminal Rel homology domain and a C-terminal IκB-like domain, similar to mammalian p100 and p105. Unlike these mammalian homologs, Relish is endoproteolytically cleaved after infection, allowing the N-terminal NF-κB module to translocate to the nucleus. Signal-dependent activation of Relish, including cleavage, requires both the *Drosophila* IκB kinase (IKK) and death-related ced-3/Nedd2-like protein (DREDD), the *Drosophila* caspase-8 like protease. In this report, we show that the IKK complex controls Relish by direct phosphorylation on serines 528 and 529. Surprisingly, these phosphorylation sites are not required for Relish cleavage, nuclear translocation, or DNA binding. Instead they are critical for recruitment of RNA polymerase II and antimicrobial peptide gene induction, whereas IKK functions noncatalytically to support Dredd-mediated cleavage of Relish.**

*Drosophila* immunity | innate immunity | NF-κB | caspase

The *Drosophila* humoral immune response is characterized by the induction of a battery of antimicrobial peptide (AMP) genes by NF-κB transcription factors (1–4). Two pathways control the activation of *Drosophila* Rel/NF-κB homologs and the induction of the AMP genes (5). The Toll pathway, which is stimulated by fungi and many Gram-positive bacteria, activates DIF and Dorsal, 2 p65-like proteins (6). However, the immune deficiency (IMD) pathway, which is triggered by Gram-negative bacteria and certain Gram-positives, activates Relish, a p100-like NF-κB precursor protein, resulting in induction of antimicrobial peptide genes including *Diptericin* (7–10).

Unlike the mammalian NF-κB proteins, Relish processing does not require the ubiquitin/proteasome pathway, but instead is an endoproteolytic, caspase-dependent event. Stimulation of the IMD pathway by Gram-negative bacteria [or DAP-type peptidoglycan (PGN)] leads to cleavage of Relish, producing an N-terminal Rel homology domain (RHD) transcription factor module that translocates to the nucleus and activates immune genes. The stable C-terminal domain (CTD) of Relish remains in the cytoplasm (11). Relish cleavage occurs after residue D545, within a typical caspase target motif (LQHDG). Death-related ced-3/Nedd2-like protein (DREDD), a *Drosophila* caspase-8 like protease, is required for Relish cleavage, AMP gene induction, and physically interacts with Relish in cell culture (12, 13). Likewise, caspase inhibitors prevent Relish cleavage and AMP induction. Together, these data suggest that DREDD is the caspase that directly cleaves Relish, although this has not yet been demonstrated. It should also be noted that DREDD has been reported to function upstream in the IMD pathway and is required for IκB kinase (IKK) activation in addition to its proposed role in cleaving Relish (14).

The *Drosophila* IKK complex is also necessary for Relish cleavage. The *Drosophila* IKK complex contains 2 subunits: a catalytic kinase subunit encoded by *ird5* (IKKβ) and a regulatory subunit encoded by *kenny* (IKKγ) (15–17). In vitro, the IKK complex directly phosphorylates Relish (15). Furthermore, *ird5* or *kenny* larvae fail to cleave Relish (13), and RNAi targeting of either IKK subunit prevents PGN-induced cleavage in S2* cells (15). Finally, the C-terminal 107 residues of Relish are required for both its phosphorylation in vitro and cleavage in cells (13), suggesting phosphorylation and cleavage are linked, but a causal relationship has not been firmly established.

In this report, we identify 2 serine residues in Relish that are phosphorylated by *Drosophila* IKKβ. These residues, serines 528 and 529, are contained in the N-terminal transcription factor module of Relish. Surprisingly, these phosphorylation sites are not required for Relish cleavage. Moreover, the IKK complex functions noncatalytically in the cleavage of Relish. Relish phosphorylation also does not control nuclear translocation or DNA binding. Instead, phosphorylation is critical for the proper transcriptional activation of Relish target genes via efficient recruitment of RNA polymerase II to the promoters of antimicrobial peptide genes.

## Results

We have previously demonstrated that 107 C-terminal residues of Relish are required for its IKKβ-mediated phosphorylation in vitro and signal-dependent cleavage in cells (13). However, when all 10 serines and threonines in this 107-aa C-terminal region were changed to alanine, Relish was still phosphorylated normally in vitro and was cleaved normally after immune stimulation in cells (Fig. S1). Thus, the C terminus of Relish is not a major target of IKK-mediated phosphorylation, and phosphorylation of this region is not required for cleavage. Instead, this region is required for the interaction between Relish and IKKβ (D.E.-H. and N.S., unpublished data).

**Relish Is Phosphorylated by IKKβ Primarily on Serine Residue(s).** To identify the amino acids on Relish that are phosphorylated by

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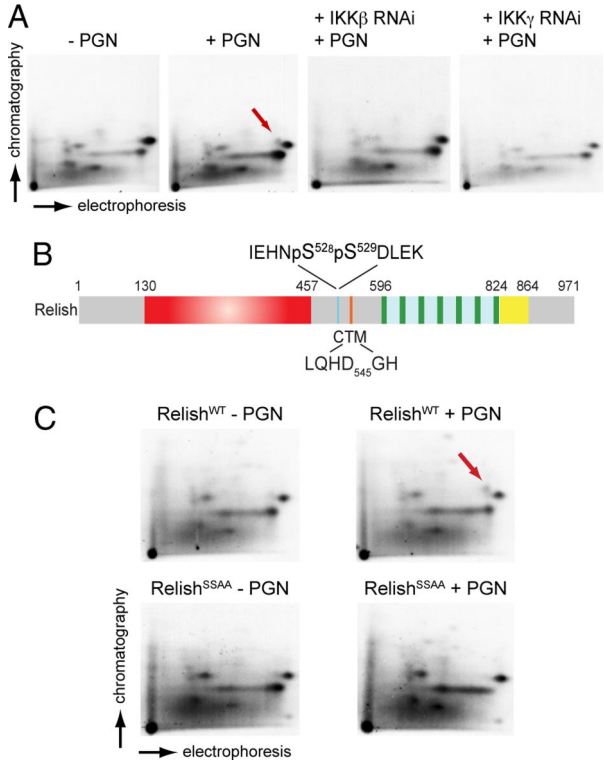


**Fig. 1.** 2D phosphopeptide mapping and mass spectrometry analysis of Relish identifies sites of signal-induced Relish phosphorylation. (*A*) S2* stable cell lines expressing wild-type FLAG-tagged Relish were radiolabeled and treated with caspase inhibitor to prevent cleavage. Relish phosphopeptides were then analyzed by 2D phosphopeptide mapping. The arrow indicates the single phosphopeptide spot that reproducibly appeared after PGN stimulation. (See also Fig. S3.) Moreover, this phosphopeptide was not detected in cells treated with RNAi targeting IKK$\beta$ or IKK$\gamma$. (*B*) Schematic diagram summarizing the phosphorylation and cleavage sites of Relish. $\mu$LC/MS/MS identified serines 528 and 529 as the in vivo immune-induced phosphorylation sites on Relish. CTM, caspase target motif. (*C*) Serines 528 and 529 are phosphorylated in vivo in a signal-dependent manner. Wild-type and SS528/529AA mutant Relish were analyzed by 2D phosphopeptide mapping. The phosphopeptide spot that appeared on PGN stimulation in wild-type Relish was not detected in the mutant form of Relish.

IKK$\beta$, we performed 2-dimensional phosphoamino acid analysis of in vitro-phosphorylated Relish. This analysis revealed that IKK$\beta$ phosphorylates Relish primarily on serine residues, with a minor 6% of phosphotyrosine and 8% of phosphothreonine (Fig. S2).

**Immune-Induced Phosphorylation of Relish Is Dependent on the IKK Complex and Other IMD Pathway Components.** To examine immune-induced phosphorylation of Relish, 2-dimensional phosphopeptide maps of in vivo-labeled Relish, before and after PGN stimulation, were generated. Because we did not know a priori which portion of Relish would be phosphorylated, Relish cleavage was inhibited with a caspase inhibitor. This analysis showed several phosphopeptide spots before immune stimulation, indicating that Relish is phosphorylated on multiple residues in a signal-independent manner. In addition, one phosphopeptide spot reproducibly appeared after PGN stimulation (arrow in Fig. 1*A* and Fig. S3). When a similar experiment was performed with the noncleavable D545A Relish, the same PGN-induced phosphopeptide spot was observed (Fig. S3*C*). To test whether this signal-induced Relish phosphorylation depends on the IKK complex, S2* cells were treated with dsRNA against IKK$\beta$ or IKK$\gamma$. RNAi treatment resulted in loss of the signal-dependent phosphopeptide spot (Fig. 1*A*). Similar phosphopeptide maps from cells treated with RNAi targeting other IMD pathway components were missing the signal-induced phosphopeptide spot (Fig. S3), whereas LacZ RNAi had no effect (Fig. S3*B*). These results demonstrate that signal-induced phosphorylation of Relish requires DREDD, *Drosophila* inhibitor of apoptosis protein 2 (DIAP2), TGF-$\beta$-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2), and the IKK complex, consistent with previous publications (14, 15, 18).

**Phosphorylation of Serine 528 and Serine 529.** To identify the sites of signal-induced phosphorylation, mass spectrometry was performed on Relish before and after PGN stimulation. Because it was not known whether phosphorylation event(s) were taking place in the N- or C-terminal products of Relish, an uncleavable version (D545A) was used in these assays. Immunoprecipitated Relish was analyzed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry ($\mu$LC/MS/MS). These data identified several sites (S32, S35, S41, S65, S464–T469) of constitutive phosphorylation, but only 2 adjacent serines, 528 and 529, were phosphorylated in a PGN-inducible manner (Fig. 1*B*). These 2 serines are just N-terminal to the cleavage site (D545) of Relish.

To confirm the mass spectrometric data, the 2D phosphopeptide maps of wild-type and SS528/529AA mutant Relish were compared (Fig. 1*C*). The signal-induced phosphopeptide spot was not detected in SS528/529AA Relish, demonstrating that serines 528 and 529 are required for the only signal-induced phosphorylation detected.

**IKK$\beta$ Directly Phosphorylates Serines 528 and 529.** To further characterize the phosphorylation status of serines 528 and 529, a phosphospecific antibody was generated. In vitro kinase assays were performed with baculovirus-produced, Ni-nitrilotriacetate (NTA)- and MonoQ-purified recombinant Relish and IKK$\beta$ wild type. An inactive version of IKK$\beta$ (K50A) was similarly produced and purified by Ni-NTA chromatography. Relish was phosphorylated by the wild-type IKK$\beta$ but not the kinase-dead IKK$\beta$ (Fig. 2*A*, lane 2 compared with lane 3, *Top*). Moreover, this phosphorylation was recognized by immunoblotting with the phospho-528/529-specific antibody.

In addition, in vitro-translated Relish proteins, both wild type and SS528/529AA mutant, were used as substrates in kinase assays with recombinant IKK$\beta$. In these assays, the SS528/529AA mutant Relish exhibited a 30% reduction in the total level of phosphorylation (Fig. 2*B*, lane 4 compared with lane 6, *Top*). The phosphospecific anti-Relish antibody also recognized the in vitro-translated and in vitro-phosphorylated wild-type Relish, but not the SS528/529AA mutant (Fig. 2*B*, lane 6, *Middle*), demonstrating that these 2 serines are direct targets of IKK$\beta$-mediated phosphorylation. Similarly in cells, overexpressed wild-type and uncleavable D545A Relish, but not the SS528/529AA mutant, was detected with the phosphospecific antibody only after immune stimulation, confirming that serines 528 and 529 are targets of signal-dependent phosphorylation in cells (Fig. 2*C*). A time course of immune stimulation was performed to characterize the dynamics of phosphorylation and cleavage of endogenously expressed Relish in S2* cells and in flies. Endogenous Relish was cleaved and phosphorylated within 1 min of PGN stimulation of S2* cells (Fig. 2*D*). Phosphorylation diminished over time, such that it was almost undetectable by 2 h. By using the phosphospecific anti-Relish antibody, cleaved and phosphorylated N-terminal Relish was also detected in extracts from whole flies within 30 min of infection with live *Escherichia coli* (Fig. 2*E*). Similar to cell culture, phosphorylation decreased over time and was almost undetectable 4 h after septic injury. In both cells and flies, phosphorylation of Relish coincided with the appearance of cleaved Relish (Fig. 2 *D* and *E*, *Lower*).

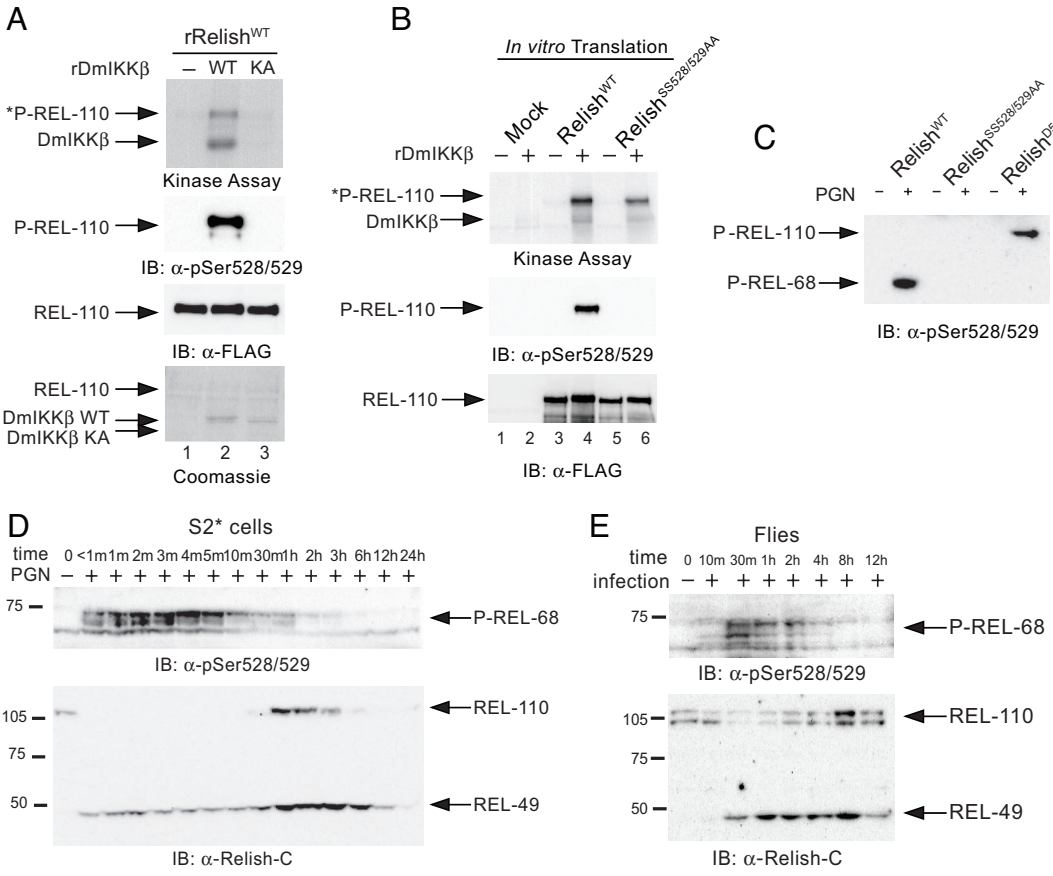


**Fig. 2.** Characterization of serine 528 and 529 phosphorylation in vitro and in vivo. (*A*) Baculovirus-expressed and purified recombinant Relish was used as a substrate in kinase reactions with baculovirus-expressed recombinant *Drosophila* IKK$\beta$ wild-type and kinase-dead K50A mutant in the presence of [$\gamma$-$^{32}$P]ATP (*Top*, lanes 2 and 3). (*B*) In a similar kinase assay, in vitro-translated wild-type and SS528/529AA versions of Relish were phosphorylated with recombinant *Drosophila* IKK$\beta$ and [$\gamma$-$^{32}$P]ATP (*Top*, lanes 4 and 6). One-third of each reaction was also immunoblotted with anti-phospho-Relish antibody, detecting serine 528/529 phosphorylation (*Middle*), whereas the other one-third was immunoblotted to confirm the presence of the Relish (*Bottom*). (*C*) Lysates from stably transfected S2* cells, expressing wild-type, D545A, or SS528/529AA mutant Relish, were analyzed by immunoblotting with anti-phospho-Relish antibody. Cells were stimulated with PGN for 10 min or left untreated, as indicated. (*D*) Rapid phosphorylation of endogenous Relish in S2* cells was monitored, with anti-phospho-Relish immunoblotting. (*E*) Similarly, rapid Relish phosphorylation was detected within 30 min of *E. coli* infection in flies.

**Epistatic Analysis of Relish Phosphorylation.** To analyze the epistatic relationship between Relish phosphorylation and IMD pathway components, RNAi was used to target most other members of the pathway. Whole-cell lysates were analyzed by immunoblotting with Relish-C antibody to monitor cleavage, and with the phosphospecific Relish antibody. Peptidoglycan recognition protein LC (PGRP-LC), IMD, Fas-associated death domain protein (FADD), DREDD, Uev1a/Ubc13, DIAP2, TAB2, TAK1, and IKK$\gamma$ were all required for phosphorylation of Relish on serines 528 and 529 (Fig. 3, *Lower*). However, RNAi against Akirin, a recently identified member of the IMD pathway, which is proposed to function downstream of Relish (19), did not prevent signal-dependent phosphorylation of Relish. Also, RNAi targeting PGPR-LC, FADD, and DREDD severely reduced Relish cleavage, but TAK1 RNAi had little effect, consistent with earlier reports (12, 13, 20). RNAi targeting of IMD, Uev1a/Ubc13, DIAP2, or TAB2 reduced but did not eliminate Relish cleavage.

**DREDD, but Not IKK-Mediated Phosphorylation, Controls Relish Cleavage.** To probe the mechanisms of Relish cleavage, we generated stable cell lines that inducibly express the caspase-8 like *Dredd*, which has been proposed to be the Relish protease. Interestingly, expression of DREDD was sufficient to cause Relish cleavage (Fig. 4*A*, lane 7, top image) but did not lead to Relish phosphorylation at serines 528/529 (Fig. 4*A*, lane 7, second image) or *Diptericin* induction (Fig. 4*A*, lane 7, bottom images). To probe the specificity of Relish cleavage by DREDD, stable cell lines similarly expressing catalytically inactive DREDD$^{C\text{-}A}$ or another *Drosophila* apical caspase, *Drosophila* Nedd2-like caspase (DRONC), were analyzed. Neither DRONC nor DREDD$^{C\text{-}A}$ cleaved Relish (Fig. S4*A*). These results suggest that DREDD specifically cleaves Relish and signal-induced phosphorylation is not required for Relish cleavage. Furthermore, Relish cleavage is not sufficient to drive *Diptericin* expression.

In vitro assays further support the model that DREDD cleaves unphosphorylated Relish. Wild-type DREDD, immunopurified from cell extracts, cleaved recombinant Relish in vitro (Fig. 4*B*). In these assays, wild-type DREDD in the presence of caspase inhibitors or catalytically inactive DREDD were unable to cleave recombinant Relish (Fig. 4*B*). To investigate the specificity of this cleavage, the same experiment was performed by using another caspase, *Drosophila melanogaster* ICE/CED-3-related protease (drICE). Recombinant drICE was unable to cleave Relish, whereas under the same conditions it efficiently cleaved poly(ADP-ribose) polymerase (Fig. S4*B*). Phosphorylation of Relish, with recombinant IKK$\beta$ before use in the in vitro cleavage reaction, did not improve the efficiency of the in vitro cleavage reaction (data not shown). Furthermore, mutation of serines 528 and 529 to alanine did not prevent Relish cleavage,

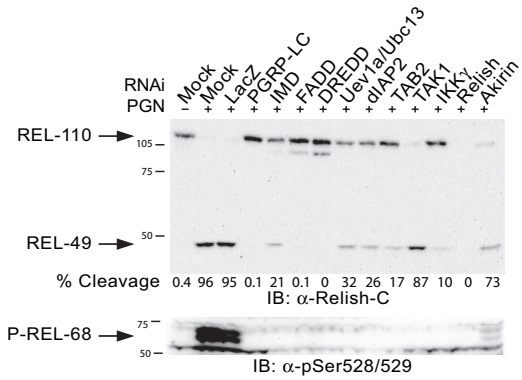


**Fig. 3.** Epistatic relationship between Relish and IMD pathway components. S2 cells were treated with RNAi against IMD pathway components and cell lysates were analyzed by immunoblotting with anti-Relish-C antibody (*Upper*) and anti-phospho-Relish antibody (*Lower*). RNAi targeting of all of the IMD pathway components, except Akirin, prevented signal-dependent phosphorylation of Relish.

demonstrating that phosphorylation of serines 528 and 529 is not required for signal-dependent cleavage of Relish (Fig. 4*C*). In fact, phosphorylation at serines 528/529 occurs in the absence of cleavage (Fig. 2*C*).

**Phosphorylation of Relish Is Required for Immune-Induced Gene Expression.** To probe the role of Relish phosphorylation in controlling AMP gene expression, the immune response of cells stably expressing wild-type or mutant Relish was compared. In cell lines expressing the SS528/529AA-substituted Relish, *Diptericin* and *Attacin* induction was markedly inhibited (Fig. 5*A*). To investigate the role of IKK$\beta$-mediated phosphorylation in vivo, we generated 2 rescue strains. These strains include loss-of-function IKK$\beta$ alleles ($ird5^1$) but carry transgenic version encoding either a wild-type kinase or a catalytically inactive K50A version. Strikingly, IKK$\beta$ catalytic activity was not required for infection-induced cleavage of Relish (Fig. 5*B*, *Top*). This is consistent with the finding that serines 528 and 529 are not required for Relish cleavage (Fig. 4*C*). However, the induction of *Diptericin* and *Attacin* expression was markedly reduced in absence of kinase activity (Fig. 5*B*, *Bottom*). In an earlier article, we reported that very strong overexpression of IKK$\beta$-K50A weakly blocked Relish cleavage in cultured cells (15). This very high level of IKK$\beta$-K50A expression was required to inhibit Relish cleavage and is likely the reason for the apparent contradiction with our current study, which was performed in vivo with only modest expression of IKK$\beta$-K50A.

To further examine how the phosphorylation of serines 528 and 529 affect the function of Relish downstream of cleavage, nuclear translocation and DNA binding were analyzed. Nuclear translocation of YFP-tagged Relish was examined by using confocal microscopy. Similar to wild-type Relish, SS528/529AA mutant was mostly cytoplasmic in unstimulated cells and translocated to the nucleus on PGN stimulation (Fig. S5*A*). To determine whether phosphorylation of serines 528 and 529 control DNA binding, chromatin immunoprecipitation (ChIP) was used, with primers amplifying the region of the *Diptericin* promoter containing 2 well-characterized $\kappa$B binding sites (21–24). Both wild-type and mutant Relish bound the *Diptericin* promoter with similar efficiencies (Fig. S5*B*, *Upper*). DNA binding was also analyzed in vitro in a pull-down assay by using biotinylated double-stranded oligonucleotides corresponding to the $\kappa$B sequences of the *Diptericin* gene. Both wild-type and mutant Relish bound to $\kappa$B sequences showing that serines 528 and 529 are not required for DNA binding (Fig. S5*B*, *Lower*).

Together, these results suggest that phosphorylation of serines 528 and 529 regulate Relish transcriptional activity, rather than cleavage, nuclear translocation, or DNA binding. To examine the interaction between Relish and components of the transcriptional machinery, ChIP assays were performed with anti-RNA polymerase II antibody (8WG16). The 8WG16 monoclonal antibody preferentially recognizes the unphosphorylated form of the RNA polymerase II and therefore monitors the preinitiation complex (25–27). The recruitment of unphosphorylated RNA polymerase II to the *Diptericin* locus was monitored by using 7 primer pairs ranging from −1.8 kbp upstream of the transcription start site to +5.5 kbp, which is downstream of both *Diptericin* and *Diptericin-B* (Fig. 5*C*). RNA Pol II ChIP assays were performed either before or 10 min after immune stimulation, from cells expressing either wild-type Relish or the SS528/529AA mutant (Fig. 5*D*). RNA polymerase II was robustly

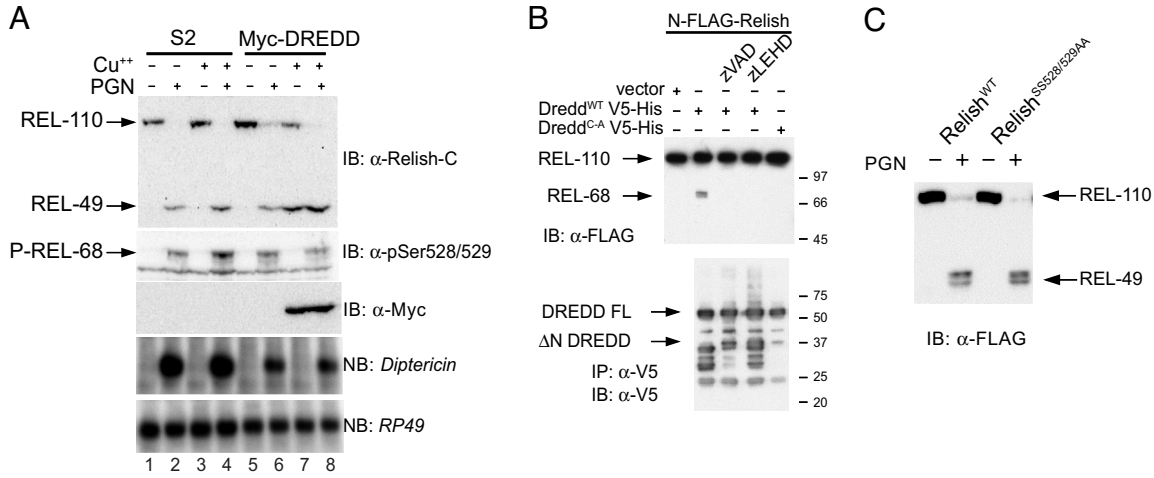


**Fig. 4.** The *Drosophila* caspase DREDD cleaves Relish. (*A*) Myc-tagged DREDD was overexpressed by using a copper-inducible promoter and lysates were analyzed for Relish cleavage and phosphorylation by immunoblotting (top 2 images). DREDD expression was monitored with anti-Myc immunoblotting (middle image), whereas Northern blot analysis was used to monitor *Diptericin* and RP49 expression (bottom 2 images). (*B*) Active DREDD was isolated from S2 cell lysates with anti-V5 beads and incubated with recombinant FLAG-Relish. The N-terminal Relish cleavage product was detectable, by anti-FLAG immunoblot, with the wild-type DREDD but not in the presence of caspase inhibitors or catalytically inactive DREDD-C408A. (*C*) SS528/529AA Relish was efficiently cleaved after PGN stimulation of S2* cells, as monitored by immunoblotting.

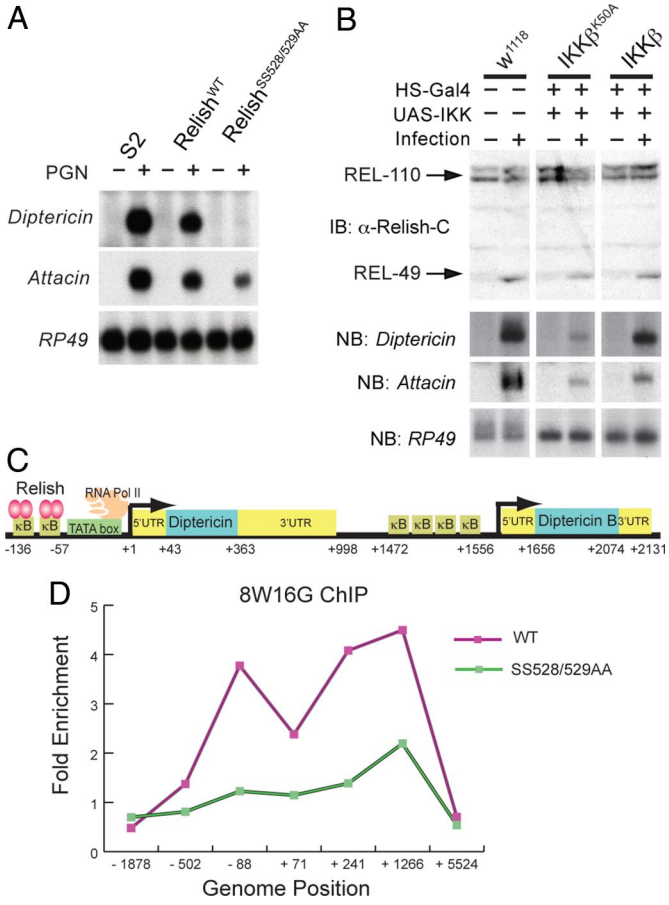


**Fig. 5.** Phosphorylation of Relish is required for IMD signaling. (*A*) S2* cells stably expressing either mutant or wild-type Relish were stimulated with *E. coli* PGN. Induction of *Diptericin* and *Attacin* was monitored by Northern blot analysis. In cell lines expressing the SS528/529AA mutant Relish, antimicrobial peptide gene induction was greatly reduced. (*B*) In transgenic rescue flies, Relish was cleaved in response to infection even when only catalytically inactive IKK, IKK$\beta^{K50A}$, was present (top image). However, Relish cleavage in the absence of phosphorylation did not support robust *Diptericin* and *Attacin* induction (bottom images). (*C*) Schematic representation of the *Diptericin* locus shows the genomic region containing *Drosophila Diptericin* and *Diptericin-B* genes. Upstream of *Diptericin* are 2 well-characterized κB motifs, where Relish binds. The *Diptericin-B* promoter also has putative κB sites. This diagram is not drawn to scale. (*D*) RNA Pol II recruitment to the *Diptericin* locus as assayed by ChIP with 8WG16, recognizing the unphosphorylated carboxyl-terminal repeat domain (CTD) of the largest subunit of RNA Pol II. Fold enrichment was calculated and plotted in the *y* axis. The *x* axis is a nonlinear representation of the 7 primer pairs that were used to monitor RNA Pol II recruitment across the *Diptericin* locus. The primer numbers indicate the center of each ≈250-bp amplicon.

recruited to the *Diptericin* and *Diptericin-B* promoters in wild-type Relish-expressing cells, but in the SS528/529AA-expressing cells RNA Pol II recruitment was greatly diminished.

## Discussion

The data presented here suggest a new model for the regulation of Relish activity. In this model, Relish is controlled by 2 distinct mechanisms, both of which signal downstream of the receptor PGRP-LC. One arm controls the cleavage of Relish and requires IMD, FADD, DREDD, and the IKK complex. The other arm controls Relish phosphorylation through TAK1 and the IKK complex. Robust induction of antimicrobial peptide expression requires that both mechanisms of control are fully active; Relish must be cleaved and phosphorylated.

Phosphorylation of Relish is critical for signal-dependent transcriptional activation of target genes. By using mass spectrometry and in vitro kinase assays, we identified serines 528 and 529 as targets of IKK$\beta$ phosphorylation. These serines are phosphorylated rapidly, in cell lines and flies, after immune challenge. Mutation of these residues to alanine resulted in a protein that acted as dominant negative in cell culture, inhibiting the PGN-induced expression of antimicrobial peptide genes *Diptericin* and *Attacin*. A recent study reported that ectopic expression of REL-68 (the N-terminal portion of Relish) was not sufficient to drive the expression of the antimicrobial peptide genes *Attacin* and *Cecropin* (28). Because REL-68 is not expected to be phosphorylated, these results support our conclusion that phosphorylation is critical for Relish-mediated transcriptional activation of AMP genes. However, this article also reported that REL-68 was able to induce *Diptericin*, which appears to contradict our findings. In these experiments, transgenic REL-68 is overexpressed, which may contribute to these confusing results.

Further supporting the conclusion that Relish phosphorylation is critical, kinase-dead IKK$\beta$ transgenic rescue supported only very weak induction of AMP genes in flies. In these experiments, Relish is expressed at normal levels. Surprisingly, serines 528 and 529, and IKK$\beta$ catalytic activity itself, were not required for signal-dependent Relish cleavage. Serines 528 and 529 were also not essential for nuclear translocation or DNA binding. Instead, ChIP experiments show that these serines are required for the efficient recruitment of RNA Pol II to the *Diptericin* locus.

These ChIP assays used the 8WG16 mAb, which preferentially recognizes unphosphorylated CTD repeats of the largest subunit of RNA Pol II. The unphosphorylated CTD is associated with the preinitiating RNA Pol II complex recruited to promoters (29). Thus, our results argue that phosphorylation of Relish on serines 528 and 529 is required for efficient recruitment of RNA Pol II to the *Diptericin* and *Diptericin-B* promoters. An alternate possibility, suggested by recent findings on gene regulation in *Drosophila*, is that phosphorylated Relish could stimulate elongation from paused RNA Pol II (30–32). However, a genome-wide analysis of promoters containing stalled RNA Pol II has found that many *Drosophila* antimicrobial peptide genes are not sites of paused RNA Pol II (33). The use of 8WG16 in the experiments presented here, further argues that phosphorylation of serines 528 and 529 does not modulate RNA Pol II pausing but instead regulates polymerase recruitment to the preinitiation complex at the *Diptericin* locus. The exact mechanism by which phosphorylation of serines 528 and 529 affect RNA Pol II recruitment remains to be elucidated. It may involve interaction with coactivators, such as components of the mediator complex, or it may involve the recently discovered IMD component Akirin, which is argued to function in the nucleus, downstream of Relish (19).

In this report, we also provide further supporting evidence that DREDD may be the caspase that directly cleaves Relish. Here, we show that overexpression of DREDD is sufficient to cause Relish cleavage. Relish cleavage required catalytically active DREDD and expression of another apical caspase, the caspase-9 like DRONC, did not generate cleaved Relish. Interestingly, DREDD-mediated Relish cleavage did not lead to Relish phosphorylation and was not sufficient to drive *Diptericin* expression. Furthermore, immunopurified DREDD, but not drICE, cleaved Relish in vitro, albeit not very efficiently. The poor efficiency of Relish cleavage, in vitro, may be due to the highly oligomeric state of purified Relish and/or the low activity of DREDD, which has proven to be very difficult to produce in an active form. We also found that a biotinylated peptide with the Relish cleavage site bound active DREDD; although strong evidence for a direct interaction, this assay is not particularly

specific. Together, these data strongly suggest that DREDD directly cleaves Relish, but we cannot yet conclude with certainty that other proteases, such as an effector caspase, are not involved.

In addition to DREDD, Relish cleavage also requires both IKK subunits, as shown here and previously (13, 15). However, Relish cleavage does not require catalytically active IKK$\beta$. Delaney et al. (20) showed that TAK1 is not required for Relish cleavage. Because TAK1 is required for the immune-induced activation of the IKK kinase (34), this result is consistent with our data indicating that IKK catalytic activity is not involved in Relish cleavage. Instead, IKK complex may function as a scaffold or adaptor, but not as a kinase, in controlling the cleavage of Relish.

Taken together, the data presented here demonstrate that Relish is regulated by 2 distinct mechanisms. Relish is probably cleaved by DREDD and phosphorylated by the IKK complex. These 2 regulatory mechanisms appear to be independent, because phosphorylation can occur without cleavage, and vice versa, although they are both triggered by PGN stimulation of the receptor PGRP-LC. Surprisingly, the IKK complex also plays a role in the cleavage of Relish, but not through its kinase activity. Instead, IKK-mediated phosphorylation of Relish on serines 528 and 529, within its N-terminal transcription factor module, is necessary for transcriptional activation of target genes.

## Materials and Methods

**Two-Dimensional Phosphopeptide Mapping.** S2* stable cell lines that express wild-type or mutant FLAG-tagged Relish were treated with 1 $\mu$M ecdysone for 24 h, and then radiolabeled with [$^{32}$P]orthophosphate for 8 h in phosphate-free medium supplemented with 0.2 mM phosphate buffer. The cells were treated with 0.1 mM caspase inhibitor z-VAD (OMe)-FMK (Calbiochem) for 20 min to prevent Relish cleavage. After 10 min of PGN stimulation, Relish was immunoprecipitated, resolved by SDS/PAGE, and transferred. Relish was excised from the PVDF membrane and digested with trypsin (Promega). The resulting peptides were resolved on thin-layer cellulose (TLC) plates (first dimension, thin-layer electrophoresis at pH 1.9; second dimension, TLC in 1-butanol, 37.5%; pyridine, 25%; acetic acid, 7.5%) and analyzed by autoradiography (35).

**Mass Spectrometry.** An uncleavable form of FLAG-tagged Relish, the D545A mutant, was immunoprecipitated from stable cell lines with anti-FLAG M2 agarose beads (Sigma), separated by SDS/PAGE, and stained with Coomassie blue. The Relish band was excised, digested with trypsin, and analyzed by $\mu$LC/MS/MS on a Thermo LTQ linear ion trap mass spectrometer.

**In Vitro Cleavage of Relish.** Cells were transfected with *Dredd* wild-type or C408A construct and lysed in nonionic detergent buffer. Then DREDD-V5 was bound to V5-agarose beads (Sigma) and incubated with 50 ng of recombinant FLAG-Relish in 20 $\mu$L of buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 5% glycerol, 1 mM DTT) for 1 h at room temperature. Beads were boiled in loading buffer and the supernatant loaded for immunoblot analysis. Expression and purification of Relish was described in ref. 15.

For more standard techniques, see detailed *SI Materials and Methods* online.

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